Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 215-221

DNA-METHYLATION OF THE E-SELECTIN PROMOTER REPRESSES NF-κB TRANSACTIVATION

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Received May 24, 1993

E-selectin is an adhesion molecule transiently and specifically expressed on endothelial cells upon stimulation with cytokines. We wished to determine whether methylation could play a role in cell-type specific expression of this gene. We found that the E-selectin promoter in cultured endothelial cells is under-methylated in comparison with non-expressing HeLa cells. Plasmid constructs carrying a reporter driven by the E-selectin promoter and methylated *in vitro* are no longer transcribed in either an *in vitro* transcription system or in transiently transfected cells. We identified the NF-κB site in the promoter as the likely target for this methylation-mediated repression by testing a minimal promoter carrying only this and an associated element. We conclude that methylation is likely to play a role in blocking E-selectin expression in non-endothelial cells.

E-selectin, also known as ELAM-1 (for Endothelial Leukocyte Adhesion Molecule-1), is a cell surface glycoprotein expressed exclusively on endothelial cells in response to cytokines IL-1β and TNF-α (1). E-selectin adheres a subset of leukocytes and is involved in chronic inflammation; aberrant expression of E-selectin is associated with several pathological conditions such as asthma and psoriasis (2) as well as formation of metastases (3). Consistent with its critical role in leukocyte adhesion and tissue infiltration, E-selectin expression is transient, peaking at about 4 h. after IL-1 induction (4-6). The up and down modulation of E-selectin expression occurs principally by altering the gene's transcription rate (6). Moreover, both the E-selectin protein and mRNA are short-lived (*ibidem*).

The DNA sequence immediately upstream of the coding sequence has been intensively studied. We have shown that an NF-κB element within this E-selectin promoter sequence is essential for IL-1 induction (5). In addition, we identified two

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0006-291X/93 $4.00

other sequence elements and their binding factors that co-operate with NF-κB for maximal, induced E-selectin transcription (7). However, the question of how tissue-specific expression occurs remained unanswered. We have shown that E-selectin/CAT gene hybrid promoter/reporter constructs carrying up to 2.5 kbp of upstream sequence lack cell-specificity (5) and this communication). We therefore tested the hypothesis that promoter methylation plays a role in tissue-specific E-selectin expression. We show here that *in vitro* methylation of the E-selectin promoter leads to nearly complete inhibition of the IL-1 inducibility of transcription.

MATERIALS AND METHODS

Cell culture and DNA transfection were as described (5).

Plasmid constructs The construction of the 3xNFEL1/κB- and E-selectin/CAT expression vectors was described previously (5, 7). The 2.5 kb construct was derived from a 4 kbp HindIII genomic E-selectin clone. Constructs with the "G-free" cassette were derived from pC_2AT, originating from R. Roeder's lab (8).

In vitro transcription Extracts for *in vitro* transcription were prepared and used as described (9, 10).

In vitro methylation of plasmid DNA Plasmids were CpG methylated to completion using SssI methyltransferase (Biolabs, New England), following the procedure described (11) and checked with HpaII.

Genomic sequencing Genomic DNA isolation from HUVEC and HeLa cells was as described (12). The DNA was modified with hydrazine and piperidine-nicked and purified on SP6-transcribed RNA affinity columns (13). Ligation-mediated PCR was as described (14) using 5'ATCCGATTGG-CTACTGTGACAGACT and its partial complement 5'AGTCTGTCACAGTAGCCAATC for ligation and PCR. The nested promoter-specific primers used for primer extension (FP1) , PCR (FP2) and end-labelled primer extension (FP3) are shown in Figure 1.

RESULTS

DNA-methylation leads to inactivation of E-selectin promoter activity

To test the hypothesis that CpG methylation plays a role in E-selectin tissue-specific expression, we examined the effects of this modification on promoter activity. The proximal E-selectin promoter sequence sufficient for IL-1 induced transcription (5) contains three CpG dinucleotides (Figure 1, shaded boxes). Although none of these sites is located directly in elements required for promoter activity (5, 7, we decided to assay the effect of methylation on sequences adjacent to these regulatory elements. We measured the transcriptional activity of E-selectin-G-free cassette-vectors (8) *in vitro* when they were either CpG-methylated to

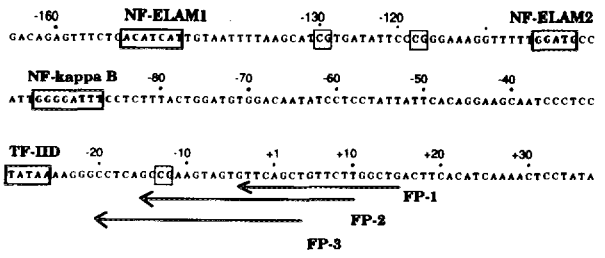

Figure 1. Proximal ELAM1/E-selectin promoter sequence. Boxes indicate functionally important elements and their binding factors as well as three CpG dinucleotides (shaded). The arrows FP-1 to -3 indicate the position and orientation of oligonucleotides used for genomic sequencing.

completion using SssI methyltransferase or untreated. We previously found that a short E-selectin promoter fragment (383 bp) is IL-1 induced to stimulate transcription both in HUVEC and HeLa cells (5). We tested the same promoter fragment *in vitro* using extracts of these two cell types (Figure 2A). While promoter activity is found without cytokine stimulation, IL-1 treatment of the cells from which extracts are made leads to an increase in the E-selectin promoter activity. Fujita *et al.* (15) have shown a similar high background for another NF-κB-dependent gene transcribed in HeLa nuclear extracts, possibly due to cytoplasmic NF-κB contamination of the extract. Nonetheless, we show here that methylation of both E-selectin constructs (Fig. 2A, lanes A and B) blocks transcription nearly to completion. This result is in stark contrast to the effect of methylation on the AdMLP construct (Fig 2A, lanes C). We next asked whether E-selectin-promoter methylation also has an effect on its *in vivo* activity. E-selectin-promoter-CAT vectors were SssI-methylated and transfected into HUVEC and HeLa cells. As seen with the *in vitro* experiments, methylation of the promoter containing vector once again leads to a strong repression of transcription *in vivo* (Figure 2B, 2C). This repression is observed for both 2.5 kbp and 383 bp of E-selectin upstream sequences driving CAT expression. We also tested whether isolated transcription factor binding sites of the E-selectin promoter are sensitive to methylation. A plasmid containing three repeats of binding sites for NF-κB and a factor we have called NF-ELAM1 (7) driving the CAT reporter gene was constructed. Interestingly, methylation of this plasmid also resulted in loss of its cytokine induced reporter activity to the same extent as the native sequence promoter fragments (Fig 2B, 2C). Previously, we have shown that the NF-ELAM1 element lacks enhancer activity on its own and must cooperate with NF-κB to achieve its effect (7). From these data we deduce that the methylation-mediated repression observed here principally operates through NF-κB, although the block cannot be through the binding site itself which lacks a CpG. Repression of NF-κB must be indirect, occurring at one of the many (a total of 217 in the vector) CpG sites found around its binding site.

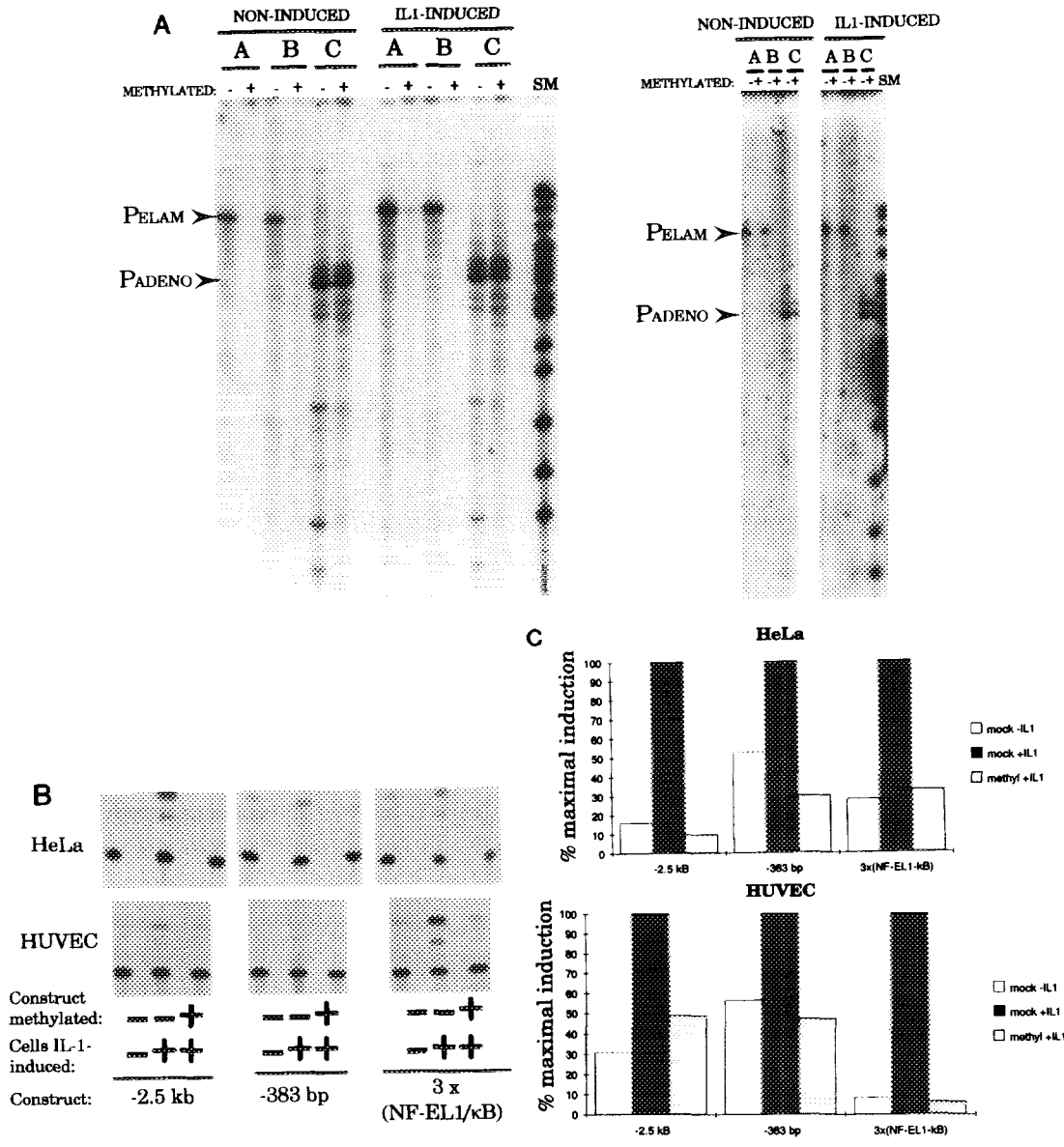

Figure 2. (A) *In vitro* transcription from methylated (+) and non-methylated (-) constructs driven by the ELAM/E-selectin promoter (constructs "A" carrying promoter fragment minus 400 to plus 1 and "B" carrying minus 400 to plus 39) or the adenovirus major late promoter ("C"). IL-1-induced or control nuclear extracts were from HeLa (left panel) or HUVEC endothelial cells (right panel). The arrowheads point to the 380 and 190 nts G-free transcripts generated by the ELAM/E-selectin and adenovirus promoter, respectively. The size markers are MspI-digested pBR322 fragments. (B) Effect of methylation on the activity of E-selectin/CAT expression vectors *in vivo*. The constructs contained 2.5 kbp or 383 bp of E-selectin promoter sequence or a trimer of the NF-ELAM1 plus NF-κB elements. (C) Graphic representation of the data shown in Figure 2B. The values are normalized for 100% maximal induction.

Methylation state of the E-selectin promoter in inducible and non-inducible cells

To establish the state of cytosine methylation in the E-selectin promoter in both inducible HUVEC and non-inducible HeLa cells, we used genomic sequencing (Figure 3). As methylated cytosine residues cannot be cleaved in this method, these residues are revealed by gaps in the ladder. A comparison of the two ladders shows that at least two cytosines in the promoter sequence are modified in HeLa DNA, but not in that of HUVECs (minus 83 and minus 118 relative to the mRNA start site). At position minus 83 the sequence is CpT while the second modification site at position minus 118 shows the dinucleotide CpG. Although methylation at the dinucleotides CpT is not as common as at CpG, this modification has been found previously in mammalian DNA (16). Close inspection of the film suggests that the cytosine of the CpG at minus 130 may also be methylated in HeLa cells. These results support our hypothesis that methylation plays a role in the *in vivo* regulation of E-selectin expression.

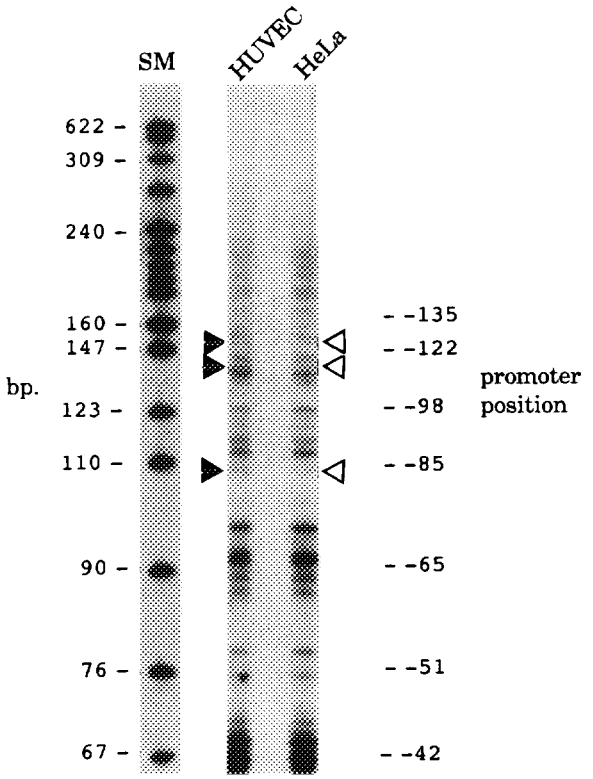

Figure 3. Genomic sequencing of the E-selectin promoter in HUVEC and HeLa cells. Filled-in arrowheads point to C-residues at positions minus 83, 118 and 130 that are visible in the HUVEC-ladder but absent or diminished in intensity in the HeLa ladder (open arrowheads). Size markers are as in Figure 2A; The corresponding promoter position of the sequencing ladder is indicated on the right.

DISCUSSION

We have demonstrated that cytokine induction of the E-selectin promoter sequences and in particular the NF-κB site can be strongly repressed by DNA methylation. Currently we cannot exclude that other important transcription-factor binding sites [e.g., NF-ELAM1/2 (7)] are similarly repressed as well. The contribution of DNA methylation to the regulation of gene expression is still poorly understood. Originally described for housekeeping genes with "CpG-rich islands", such as the *pgk-1* gene, methylation as a regulatory mechanism for CpG-poor, tissue-specific genes has been more rarely observed. Sequence analysis of 2.5 kb of the E-selectin promoter revealed an absence of CpG-rich islands (data not shown). Here we show that the E-selectin promoter--a highly tissue-restricted, cytokine-inducible promoter--is undermethylated in inducible cells compared to cells that do not express the gene. In several cases blocking by methylation of the binding site for specific transcription factors in the promoter has been suggested as the mechanism by which methylation restricts gene expression (17, 18). The evidence we present here suggests that E-selectin repression by methylation does not occur by directly preventing NF-κB binding since the site lacks a methylation signal. Potential mechanisms for methylation inactivation of transcription include the binding of proteins specific for the methylated sequence. The parent expression vector we used [pCAT-promo (Promega)] has numerous CpG pairs (in total 217). To date two ubiquitous methyl-CpG binding factors, MeCP-1 (11) and -2 (19) have been described, each having a different affinity for methylated CpG densities in DNA. The binding of one of these or another methyl-DNA recognizing protein might result in inactivation of the E-selectin NF-κB element.

We have demonstrated that the methylation block to E-selectin transcription most probably occurs through NF-κB. This factor is found to bind and transactivate an increasing number of genes. Our observation that the E-selectin NF-κB element can be strongly repressed by surrounding methylation may, in part, explain the selective role of NF-κB in the large array of different, tissue-specific genes that it affects.

Acknowledgments: We thank Dr. J. Knowles for critically reading the manuscript and F. Talabot, C. Losberger and J.-C. Ramuz for excellent technical assistance.

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